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Isolation of Yeast Catalase and Its Properties

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A highly purified yeast catalase was isolated from dried baker's yeast by the technique of fractional precipitation with ammonium sulfate and dioxane. The purified catalase solution was very stable unlike the preparations prepared by others. It did not lose the catalatic activity for several days in a refrigerator. The final preparation had a Kat. f. value of 27,250, haematin content of 0.801 per cent, and a purity of 80 per cent on electrophoresis. These properties are somewhat different from those of catalase obtained from other sources. These discrepancies were discussed in several points.

INTRODUCTION

Recently, semi-purified yeast catalase, Kat. f. value of 7,200, iron content of 0.019% has been prepared by Brown[1]. Its further purification has been prevented by the instability. However, assuming that the preparation is 20% pure, he has characterised it as follows : the pure yeast catalase would have a Kat. f. value of 36,000 and iron content of 0.095 %. The circumstances have shown the necessity for his conclusion to be ascertained, or for yeast catalase to be characterised with a more purified preparation.

EXPERIMENTALS

(A) Estimation

Catalatic activity. Assays of catalatic activity were made by the method of Euler and Josephson[2] and Sumner[3]. In case of temperature effect and inhibition studies, monomolecular rate constant k_0 values were determined by the method of Bonnichsen, Chance and Theorell[4]. The dry weight of sample was measured after drying in a weighing bottle at 105°, after dialysis for one week against glass distilled water (renewed daily) in the cold.

Haematin content. The haematin content was determined as pyridine haemochromogen by the method of Keilin and Hartree[5]. The calculation was based upon the value of $E_{557}=34.7\ mM^{-1}\cdot cm^{-1}$ [6].

Electrophoresis. Electrophoretic behaviour of the final preparation was observed in a Tiselius apparatus using a semi-micro cell (Hitachi, L form).

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(B) Purification

Ten kg. of fine ground dried yeast (Oriental Yeast Co.) was stirred into 30 l. of 0.066 *M* phosphate buffer, pH 7.0, for 2.5 hours at room temperature. The suspension was filtered with a large Büchner funnel in aid of 50 g. Hyflo super cel per liter of the suspension. The filtrate, 19 l. in total, Kat. f. of about 20, was saturated to 65% with powdered ammonium sulfate. After standing in a refrigerator overnight, the precipitate was collected and dissolved in 3.3 l. of cold distilled water. Two-tenth volume of 1.6% tricalcium phosphate gel prepared by the method of Sarker and Sumner[7], was added to the solution, and the resulting cake was centrifuged off. The clear supernatant was saturated to 20% with ammonium sulfate and the precipitate was discarded. The ammonium sulfate concentration was brought up to 65% saturation and stood overnight. The precipitate was dissolved in 1.8 l. of cold water (-5°). To this solution, total volume of 2.8 l. and Kat. f. of 250, a cold (-15°) acetone was added slowly to 45% concentration, and the precipitate was centrifuged down instantaneously at 0°. The precipitate was dispersed into 1.25 l. of cold water and stirred vigorously for a few minutes. After an insoluble protein was centrifuged off, the supernatant was saturated to 40% with ammonium sulfate, and stood for half an hour. The precipitate obtained by centrifugation was dissolved in 250 ml. of cold distilled water, which had a Kat. f. value of 4,500. The solution was diluted to give an optical density of 2.0 at 410mμ. Eighty % dioxane was added slowly into the diluted enzyme solution at 0° to 2° until dioxane concentration to be 33%. After 20 hours, the precipitate was centrifuged off, and the dioxane concentration in the supernatant was brought up to 43%. After 1.25 hours correctly, the precipitate was centrifuged down for 15 minutes at 4,000 r. p. m., and dissolved in 5 ml. of 0.066 *M* phosphate buffer, pH 6.8. This preparation, a deep brown color, had a high activity, Kat. f. value of 27,250. It was so stable that no loss of activity was observed in refrigerator for several days. The purification procedure is shown in Fig. 1 as a scheme. An attempt to crystallize this preparation met no success.

(C) Properties of Highly Purified Enzyme

(1) Absorption spectrum. The final preparation was diluted with water to fifteen times. Aliquot of the solution, 2.15 ml., was filled up to 6 ml. with 0.066 *M* phosphate buffer, pH 6.8. Then, the sample of free catalase and cyanide complex were prepared by the addition of 0.5 ml. of phosphate buffer or 0.1 *M* potassium cyanide (dissolved in 0.3 *M* potassium dihydrogen phosphate) into 2.5 ml. of the above diluted catalase solution. The absorption spectra are shown in Fig. 2. The ratio E_{280}/E_{405} in the free catalase was 1.79.

(2) Haematin content. An aliquot of five times diluted final preparation, 2.28 ml., was treated with 5 ml. of 0.1% of hydrochloric acid in acetone in the cold. After centrifugation, acetone was removed from the supernatant by introducing a stream of air. A brown precipitate was separated from a colorless aqueous layer. The precipitate was dissolved in 1.4 ml. of pyridine. To

Dried Baker's Yeast 10 kg.
- —grind
- —add 30 l. of 0.066 *M* phosphate buffer, pH 7.0
- —stirr for 2.5 hrs. at room temp.
- —filter on Büchner funnel

Clear Extract 19 l., Kat. f. 20.0
- —add solid Am_2SO_4 to 65% saturation
- —stand overnight in an ice-box

Precipitate
- —dissolve in 3.3 l. of cold distd. water
- —add 0.2 vol. of 1.6% tricalcium phosphate gel
- —centrifuge

Supernatant
- —add solid Am_2SO_4 to 20% saturation
- —centrifuge

Supernatant
- —add solid Am_2SO_4 to 65% saturation
- —filter

Precipitate
- —add 1.25 l. of cold water
- —homogenize in Waring blender
- —centrifuge

Supernatant
- —add solid Am_2SO_4 to 40% saturation
- —centrifuge

Precipitate
- —dissolve in 250ml. of cold distd. water
- —centrifuge

Clear Supernatant 300ml., Kat. f. 4,500
- —dilute with cold distd. water to give $E_{410}=2.0$
- —add 80% dioxane to 33%
- —stand overnight in an ice-box
- —centrifuge

Supernatant
- —add 80% dioxane to 43%
- —stand for 1.25 hrs. at 0°
- —centrifuge

Brown Precipitate
- —dissolve in 0.066 *M* phosphate buffer, pH 6.8

Catalase Solution 6.2 ml., Kat. f. 27,250

Fig. 1. Scheme for the purification of baker's yeast catalase.

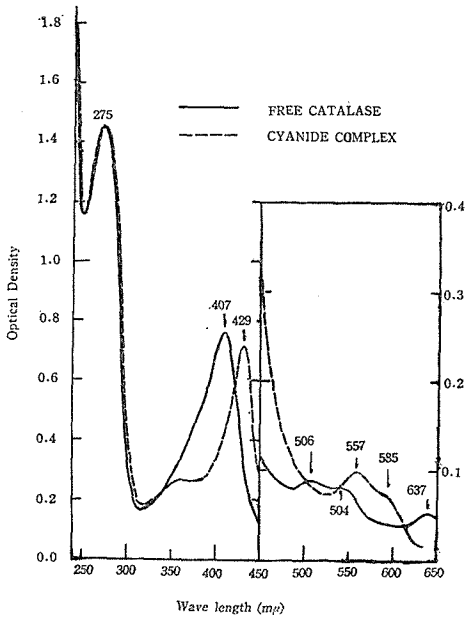

Fig. 2. The enzyme has a Kat. f. value of 27,250, and its concentration is 245 mg. per ml. Free catalase is faint orange color, and cymplex is deep yellow orange color.

this pyridine solution were added 0.3 ml. of 1 *N* sodium hydroxide and 1.3 ml. of water. The mixture showed a sharp absorption at 557 mμ by the addition of sodium dithionite. These facts indicated that the prosthetic group of yeast catalase is protohaemin and no detectable bile pigment is present. Haematin content calculated from the extinction at 557 mμ was 0.801%. This preparation was about 80% pure, as indicated in the following electrophoretic analysis. Therefore the pure yeast catalase would have a haematin content of about 1.00%.

(3) **Iron content.** One ml. of the final preparation was digested with 1 ml. of conc. sulfuric acid and 3 drops of 30% hydrogen peroxide over a flame. After cooling, to the digested solution were added 0.2 ml. of 20% sulfosalicylic acid and a few drops of conc. ammonia, and then filled up to 5 ml. with water. An extinction at 424 mμ of this sample was compared with the standard solution (10γ iron/ml.) by the Beckman spectrophotometer. The preparation contained 0.073% of iron, and was nearly equal to that calculated from haematin content.

(4)**Electrophoretic behaviour.** Twelve-tenth ml. of the final preparation was dialyzed against 0.05 *M* phosphate buffer, pH 7.05, ionic strength 0.11, for 72 hours at 5°. Electrophoretic run was made for 80 minutes in the Tiselius apparatus. The electrophoretic pattern presented in Fig. 3 showed a heterogeneity and purity of 80%. Five per cent of the total activity was lost during electrophoresis probably due to standing for long time upon 20°.

(5) **Effects of inhibitors.** A mixture of 1 ml. of dilute enzyme solution and

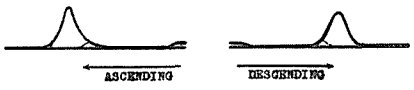

Fig. 3. Electrophoretic pattern of purified yeast Catalase. Medium ; 0.05 *M* phosphate buffer, pH 7.05, μ=0.11, field strength ; 4.124 volt/cm., temp. ; 20°, enzyme concentration ; about 0.6%.

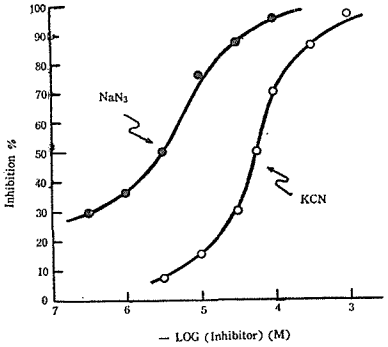

Fig. 4. Effects of cyanide and azide on the activity of purified yeast catalase.

17 ml. of 0.1 *M* phosphate buffer, pH 6.8 containing a certain concentration of potassium cyanide or sodium azide was incubated for 5 minutes at 2°. The catalatic reaction was started by the addition of 2 ml. of 0.1 *N* hydrogen peroxide, and monomolecular rate constant was calculated. Fig. 4 shows the plots of inhibition degree *versus* inhibitior concentration. A half inhibition of the catalati cactivity was produced by a 5.6×10^{-5} *M* of cyanide or 3.0×10^{-6} *M* of azide.

(6) Effects of temperature. The crude enzyme solution, Kat. f. value of 4,500, was diluted to the concentration of 0.05 mg. per ml. with 0.066 *M* phosphate buffer, pH 6.8, and incubated at 0, 10, 20, 25, or 30°. After 10 or 20 minutes, the each enzyme solution was rapidly cooled, and then its residual activity was measured. The results are shown in Table 1.

The enzyme was inactivated above 20° under these conditions. Arrnenius plots of 0, 3, 6, 5, 11.5, and 21°, are shown in Fig. 5. Activation energy was calculated as 2,150 to 2,200 cal. mole^{-1}. This value is higher than that of horse liver[8] or erythrocyte[4] or bacterial catalase[9].

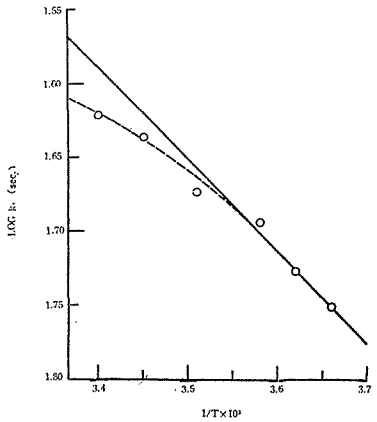

Fig. 5. An Arrrhenius plots for the effect of temperature upon the yeast catalase-hydrogen peroxide reaction.

Table 1. Thermal inactivation of yeast catalase.

Incubation time	Incubation temperature				
	0°	10°	20°	25°	30°
min.	Residual Activity in %				
10	100	100	95.4	92.5	85.7
20	100	100	93.1	87.4	74.8

Enzyme was incubated in 0.066 *M* phosphate buffer, pH 6.8, at each temperature for 10 or 20 minutes, and then its residual activity was measured at 2°.

DISCUSSION

The enzyme concentration and standing time in dioxane fractionation are the key points in the procedure of purification. Provided the enzyme solution is left standing in dioxane solution of the concentration higher than 43% or longer time than 1.25 hours, the enzyme deposits together with yellowish impure proteins. An attempt to purify by the calcium phosphate gel adsorption or ethanol fractionation procedure has been unfavorable. The highly purified preparation obtained in this procedure has been so stable as shown in the electrophoretic experiment, therefore the instability demonstrated by Brown[1] would be attributable to the inadequacy of the purification procedure. From the present data, the pure yeast catalase would have a haematin content of 1.00% and Kat. f. value of 34,000, and consists of four haematin per molecule.

The ratio E_{405}/E_{280} of the pure enzyme are calculated to be 0.70 from the absorption spectrum of this final preparation. This ratio differs greatly from that of horse liver[10], or erythrocyte catalase[11]. This discrepancy could be explained as the contamination with the substances which have great absorbing power, or as the characteristics of protein moiety which shows a high extinction at 270mμ to 280mμ. The molar extinction of haematin ($=79.3\times10^7$) of this preparation in Soret region is inferred to be proper, since it coincides with that determined with horse liver[10]. The ratio of E_{405}/E_{280} of pure yeast catalase can not be calculated from the data of this final preparation. Although Brown has suggested that the ratio E_{405}/E_{280} of pure yeast catalase is 1.08, investigating the absorption spectrum of his preparation of Kat. f. 8,000, or purity of 20%, it can not be submitted. Since his preparation has been contaminated, as he has pointed out, with some flavoprotein, which should show absorbency to some degree at 260 to 270mμ, the ratio E_{405}/E_{280} of these inappropriate preparations is unreasonable.

It has been shown by Pinsent[12] that the animal liver and blood catalase have a Kat. f. value of 55,000 to 60,000 per 1% of haematin. It still remains to be explained why a Kat. f. value of the yeast catalase is lower than those. It can be taken into consideration that a catalatic activity varies with the configuration of protein moiety.

Recently, the role of catalase in biological peroxidation and oxidation has been reported[13]. It is a most interesting problem for the authors that ribose-5-

phosphate, which is known as a participant in nucleic acid metabolism, is oxidized in the presence of catalase[14]. It seems likely that catalase *in vivo* plays a certain still unknown part other than mere catalatic function, and especially, yeast catalase has a connection with nucleic acid metabolism.

SUMMARY

(1) The highly purified catalase has been isolated from baker's yeast by the method of ammonium sulfate and dioxane fractionation.

(2) The preparation thus obtained was very stable and has a Kat. f. value of 27,250, haematin content of 0.801% and purity of 80% electrophoretically. Therefore, the pure yeast catalase would have a Kat. f. value of 34,000 and 1.00% of haematin, keeping the approximate value of the ratio of E_{405}/E_{280}.

(3) It has been discussed that a Kat. f. value of yeast catalase shows a lower one.

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